The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity *in vivo*

ZHI-YONG YANG[1,2], NEIL D. PERKINS[1,2], TAKESHI OHNO[1,2], ELIZABETH G. NABEL[2] & GARY J. NABEL[1,2]

[1]*Howard Hughes Medical Institute,* [2]*University of Michigan Medical Center, Departments of Internal Medicine and Biological Chemistry, Room 4520, MSRB I, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0650, USA*
Correspondence should be addressed to G.J.N.

The *p21* gene encodes a cyclin-dependent kinase inhibitor that affects cell-cycle progression, but the potential of this gene product to serve as a tumour suppressor *in vivo* has not been established. In this report, we show that the growth of malignant cells *in vitro* and *in vivo* is inhibited by expression of *p21*. Expression of *p21* resulted in an accumulation of cells in G0/G1, altered morphology, and cell differentiation, but apoptosis was not induced. Introduction of *p21* with adenoviral vectors into malignant cells completely suppressed their growth *in vivo* and also reduced the growth of established pre-existing tumours. Gene transfer of *p21* may provide a molecular genetic approach to arresting cancer cell growth by committing malignant cells irreversibly to a pathway of terminal differentiation.

The assembly and activation of specific cyclin/cyclin-dependent kinase (CDK) complexes regulate a series of checkpoints that determine cell-cycle progression[1–4]. These checkpoints are controlled by the synthesis and proteolysis of cyclins and phosphorylation by CDKs. In addition, regulation of cyclin/CDK activity is achieved through the CDK inhibitory proteins (CKIs), which bind and inactivate CDK complexes[5,6]. The definition of cell-cycle regulatory proteins has been greatly facilitated by studies of mutant yeast strains with abnormalities related to cell proliferation. Among the gene products defined in yeast are FAR1 and SIC1 (p40) (refs 6, 7), whose mammalian homologue, p21, alters the activity of cyclin-dependent kinases and is implicated in cell-cycle progression and senescence[5,6,8,10].

p21 was initially characterized as an inhibitor of all cyclin-dependent kinases[5,6,9,10], although it has been shown subsequently to exist also in catalytically active complexes[8]. p21 is one of several CDK inhibitors, which now include the p21 homologous protein, $p27^{Kip1}$ (refs 11, 12), $p16^{INK4}$ (ref. 13) and the related protein $p15^{INK4B}$ (ref. 14). p27 and p15 have been implicated in transforming growth factor-β (TGF-β)-induced cell-cycle arrest[11,12,14], whereas p16 is a product of the MTS1 locus, which is frequently deleted in tumour cell lines[13,14]. Binary cyclin/CDK complexes are the predominant form in certain virally transformed cell types. In normal human fibroblasts, they exist as a quaternary complex with cyclin, CDK, proliferating cell nuclear antigen (PCNA) and p21.

p21, also known as WAF1, CIP1, CAP20 or SDI1 (refs 5, 6, 10, 15–17), is induced upon DNA damage by the tumour suppressor gene *p53* (refs 15,18), and mediates G1 cell-cycle arrest[19–21], at which point DNA repair is accomplished before DNA replication in S phase. Consistent with these findings, p21 has been shown to inhibit PCNA-dependent DNA replication, but not DNA repair, *in vitro*[22]. p21 has been also shown to inhibit phosphorylation of the tumour suppressor retinoblastoma gene product[10,23]. These observations suggest that p21 might function independently as a tumour suppressor protein. We have therefore analysed the effect of p21 expression on tumour growth *in vivo*.

Gene transfer of *p21* and effect on cell-cycle progression

The effect of p21 on cell-cycle distribution was determined in tumour cell lines by infection with an adenoviral vector, ADV-p21, or a similar E1 deletion virus with no recombinant p21, ADV-ΔE1. Expression of *p21* in the adenoviral vector was regulated by the CMV enhancer/promoter and bovine growth hormone polyadenylation sequence. Expression of *p21* within a representative malignant cell line, the B16BL6 melanoma, resulted in an accumulation of cells in the G0/G1 phase of the cell cycle, suggesting arrest predominantly at the G1/S boundary (Fig. 1*a*). Recombinant p21 expression was confirmed in murine (Renca) or human (293) renal cell carcinoma lines, and the murine (B16BL6) melanoma cell line by using western blot analysis. Readily detectable protein expression from the adenoviral vector was achieved ~1 day after introduction of the gene (Fig. 1*b*, lanes 4, 5, 13, 14 versus 1–3, 10–12). In addition, a eukaryotic expression plasmid regulated by the Rous sarcoma virus (RSV) enhancer/promoter and bovine growth hormone polyadenylation site showed comparable expression in 293 cells (Fig. 1*b*, lanes 7, 9 versus 6, 8). In both cases, expression of the recombinant protein correlated with inhibition of cell division and other vectors with the same regulatory elements did not show the effects of p21 described here.

Differentiation and morphologic effects of p21

When the effect of p21 on cell growth was examined *in vitro*, tumour cells infected with ADV-p21 showed morphological changes, such as an increased nuclear-to-cytoplasmic ratio, an increase in adherence, and growth arrest, consistent with a differentiated phenotype (Figs 2, 3). Human melanoma cells,

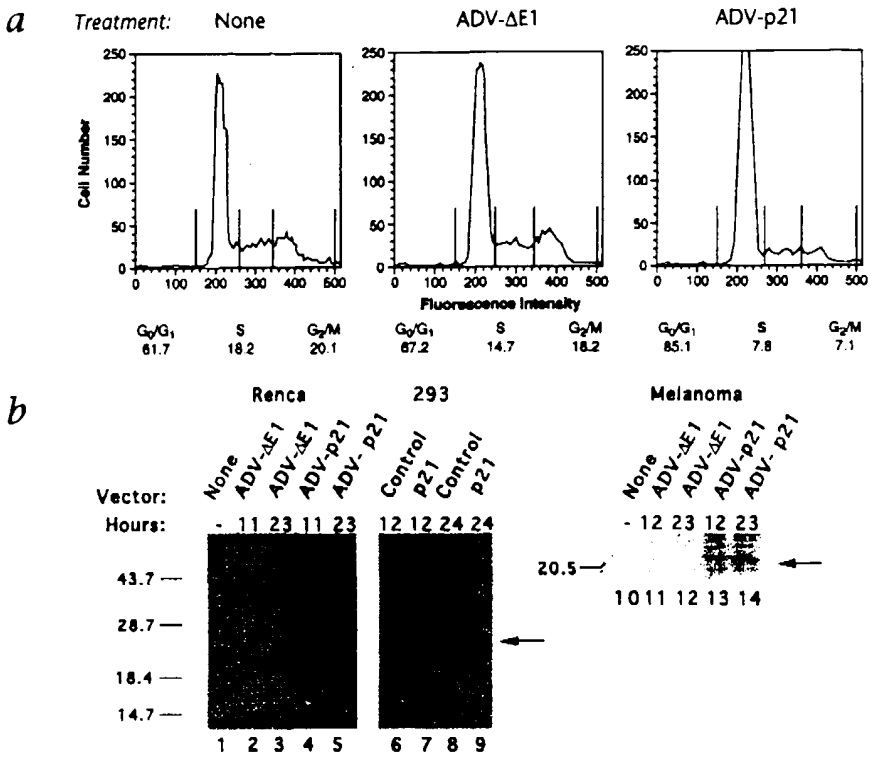

Fig. 1 Cell-cycle analysis in malignant cell lines and expression of p21. *a*, Propidium iodide staining for cell-cycle distribution in B16BL6 cells[16], a murine melanoma, infected with the indicated adenoviral vectors or PBS analysed after 48 hours. The regions between the vertical lines from left to right represent cells in G0/G1, S, and G2/M, respectively. Results are representative of three independent experiments. *b*, Western blot analysis of Renca (lanes 1–5), 293 (lanes 6–9) and B16 melanoma (lanes 10–14) cell lines transduced with the indicated adenoviral and eukaryotic expression vectors.

UM-316, showed nuclear condensation and a greater than fourfold increase in melanosome formation by electron microscopy after infection with ADV-p21 (Fig. 2; $P \leq 0.005$ by the Wilcoxon rank sum test). In these cells, an approximately fivefold increase in melanin production was observed within two days after gene transfer in cells and supernatant fractions *in vitro* (Fig. 3).

In some lines, cell death was observed to follow terminal differentiation after extended cell culture, but there was no evidence of apoptosis, as determined by the pattern of DNA fragmentation (Fig. 4*a*), propidium iodine staining or terminal deoxyribonucleotidyl transferase (TdT) immunostaining (Z.-y. Y. *et al.*, unpublished data). In addition, mixtures of uninfected and infected cells failed to show a bystander effect (Fig. 4*b*), suggesting that gene transfer and expression in recipient cells was required and that efficient infection with p21 is required to eradicate growth of established tumours.

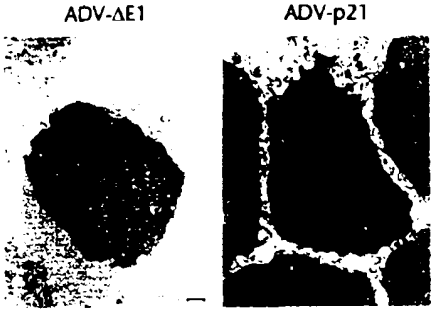

Fig. 2 Effect of p21 gene expression on melanosome formation in human melanoma cells. A human malignant melanoma line, UM-316, was infected with ADV-ΔE1(left) or ADV-p21 (right) and analysed by electron microscopy 4 days later. Arrows denote representative mature melanosome structures. Quantification of melanosome number revealed a median of 8.2 per cell for ADV-ΔE1 cells and 37.6 per cell for ADV-p21 ($P \leq 0.005$ by Wilcoxon rank sum test). (Magnification, ×3,400; bar, 2-μm segment).

Inhibition of tumour cell growth *in vivo*

To assess the effect of p21 on the growth of malignant cells *in vivo*, Renca cells were infected with ADV-p21 or an ADV-ΔE1 control, or incubated with phosphate-buffered saline (PBS), and inoculated into recipient animals. p21 expression completely suppressed the growth of tumours in all animals inoculated with 2×10^5 cells (Fig. 5*a*, *b*). Because it remained possible that expression of p21 could alter the immunogenicity of infected cells and thus work through an immune mechanism, similar studies were undertaken in CD-1 *nu/nu* immunodeficient mice. Similar inhibition of tumour growth was observed in these animals (Fig. 5*c*, *d*), consistent with a direct effect on cell proliferation.

To determine whether ADV-p21 could alter the growth of established tumours, Renca tumour nodules (~0.5 cm) were injected with PBS, ADV-ΔE1, or ADV-p21. Direct transfer of adenoviral vectors encoding a human placental alkaline phosphatase reporter into established tumours caused infection of up to 95% of cells as estimated by quantitative morphometry after five repeated daily injections of 10^9 plaque-forming units (PFU) (Z-y. Y. *et al.*, unpublished data). This treatment also inhibited tumour growth (Fig. 6*a*), and when injections were performed repetitively (five daily injections, repeated after one week), could lead to long-term cure as determined by survival (>40 days) and our inability to detect macroscopic tumour in mice with previously detectable nodules (Fig. 6*b*). In both cases these results were statistically significant (Fig. 6, legend).

Discussion

p21 affects cell-cycling and differentiation of malignant cells

In these studies, we have analysed the role of the p21 cyclin-dependent kinase inhibitor on tumour cell growth. p21 is induced by p53 (refs 5, 6, 15, 18–20) and has thus been implicated as a downstream effector of p53 tumour suppression[24]. Here, we provide the first direct demonstration that p21 expression is sufficient to produce these tumour suppressor effects *in vivo*: p21 arrests tumour

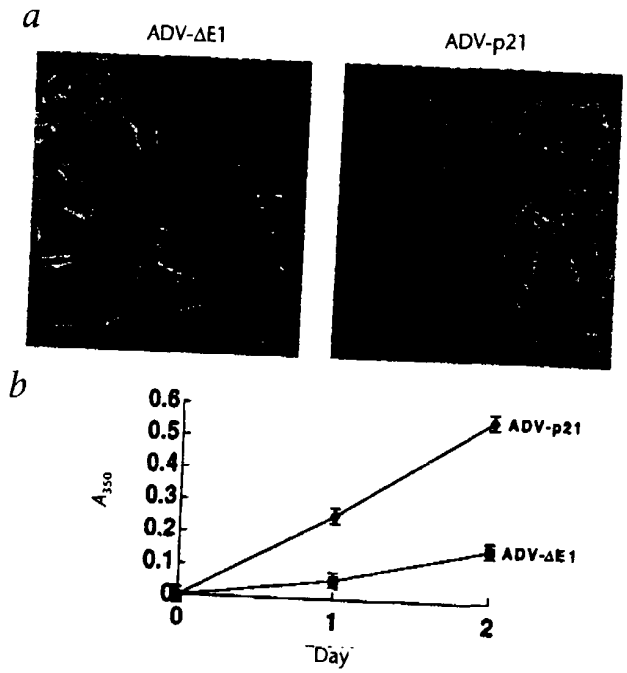

Fig. 3 Expression of melanin in UM-316 human melanoma cells in response to p21. *a*, Phase-contrast microscopy of UM-316 cells transduced with ADV-ΔE1 or ADV-p21 (magnification, ×20; bar, 16-μm segment). *b*, Colorimetric assay of melanin production[43] as a function of time in these cells reveals a statistically significant difference in melanin accumulation in the supernatant solution on day 1 ($P < 0.0009$) and day 2 ($P < 0.0001$).

cell growth by blocking cell-cycle progression, through the induction of terminal differentiation and by growth arrest.

The mechanism of p21-induced cell-cycle inhibition is not completely understood; however, it is likely that p21 inhibits the ability of specific cyclin/CDK complexes (for example, cyclin D/CDK4) to phosphorylate retinoblastoma (Rb) (refs 5, 10, 23, 25–29). Phosphorylation of Rb at the G1/S boundary leads to the release of E2F transcription factors that facilitate progression through S-phase, probably through transcriptional effects on a variety of target genes[5,30–32]. We have found that p21 expression also facilitates transcriptional activation by nuclear factor NF-κB (N.D.P. *et al.*, manuscript submitted). p21 can therefore directly influence the expression of genes, such as adhesion molecules, associated with differentiation at the same time it controls cell-cycle progression. It is therefore likely that the suppression of tumour growth and induction of the differentiated phenotype arises from altered patterns of gene expression, mediated in part by NF-κB, resulting from p21-induced transcriptional regulation leading to terminal differentiation and growth arrest. An analogous link between CKIs and transcriptional regulation has been observed in yeast. The PH081 protein, for example, shows homology with the mammalian $p16^{INK}$ CKI, and inhibits phosphorylation of the transcription factor PH04 by the cyclin/CDK-like complex PH080–PH085, resulting in stimulation of PH05 gene expression[33,34], which is involved in the regulation of phosphate metabolism.

Therapeutic potential of p21 in malignancy

Previous attempts to induce antitumour effects through induction of terminal differentiation have involved the use of cytotoxic drugs or hormones[35–38], which have had variable success. This variability may be due to heterogeneity for the targets of such drugs in tumours or insensitivity of signals downstream of this interaction. In contrast to such drugs which act indirectly on proliferation, it appears that p21 acts directly on one of the final effectors that modulate cell proliferation. Expression of this gene product in several tumour types appeared to inhibit cell-cycle progression and to stimulate differentiation.

The present study suggests that molecular genetic intervention with p21 can consistently exert effects in diverse tumour types and that the pathway leading to terminal differentiation in tumour cells can proceed irreversibly *in vivo*. Because no adverse effects have been detected *in vivo* in normal cells and tissues (Z.-y. Y. *et al.*, manuscript submitted and data not shown), these findings could be applied to clinical settings. For example, p21 could be induced indirectly, by p53-mediated induction by DNA damage, or by identification of p21 molecular agonists. The observations reported here suggest that direct gene transfer of p21 using viral vectors, such as adenoviruses or possibly adeno-associated viruses or nonviral delivery with repeated administration of DNA–liposome complexes, would reduce tumour growth. Because it may be difficult to achieve gene transfer into all cells in established tumours *in vivo*, it may prove necessary to combine gene delivery of *p21* with other genes that can affect the growth of untransduced cells, using a combination of antiproliferation and immune-activating genes. Such combined approaches have shown improved efficacy in animal models (ref. 39 and Ohno *et al.*, unpublished observations). Gene transfer of *p21*, or modulation of its activity by other

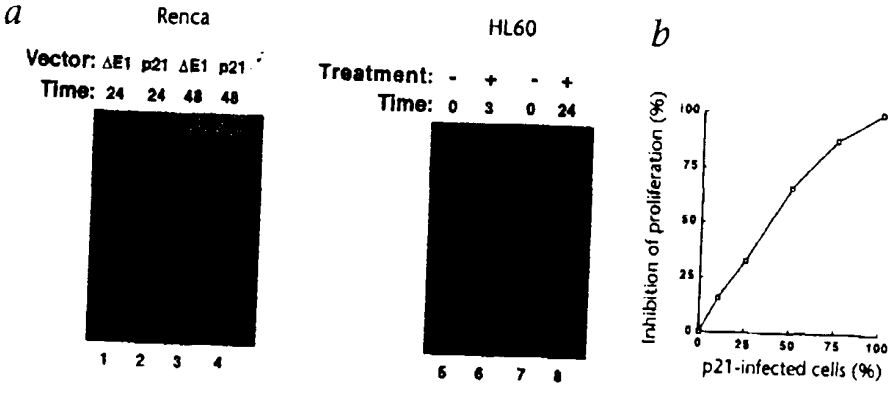

Fig. 4 Lack of evidence for apoptosis or bystander killing in ADV-p21-infected Renca cells. *a*, Renca cells were infected with ADV-ΔE1 (lanes 1, 3) or ADV-p21 (lanes 2, 4) or HL-60 cell were untreated (lanes 5, 7) or incubated with 0.2 μM camptothecin (Sigma) (lanes 6, 8), and DNA was prepared at the indicated times and analysed by agarose gel electrophoresis. The DNA ladder assay was performed as previously described[44]. *b*, Bystander killing was assessed by using a colorimetric proliferation assay as described in Methods with the indicated different ratios of ADV-p21-infected and uninfected glioblastoma cells. Similar results were observed in other cell types.

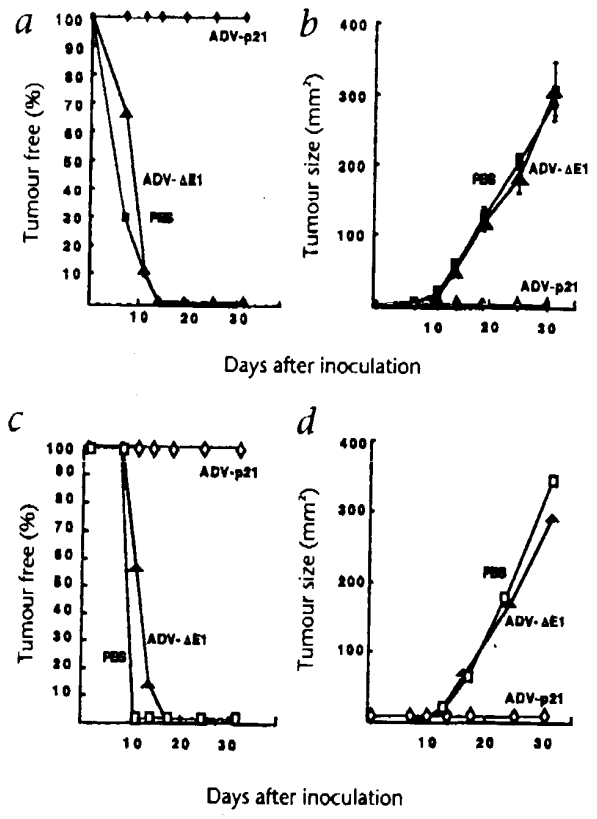

Fig. 5 Introduction of ADV-p21 into Renca tumour cells followed by *in vivo* inoculation: inhibition of tumour growth. BALB/c mice (*a*, *b*) or *nu/nu* CD-1 mice (*c*, *d*) were injected with Renca cells incubated *in vitro* with PBS (□, ■), ADV-p21(◆, ◇), or ADV-ΔE1(▲, △) at an MOI of 300. The presence of tumour (*a*, *c*) and tumour diameter (*b*, *d*) were evaluated.

means, may therefore be of potential utility in the treatment of malignancy.

Methods

Cell-cycle analysis. Cells were infected at an MOI of 200–300 with the ADV-ΔE1 or ADV-p21 vectors or transfected with the p21 expression vector by DNA–liposome complexes. The cells were infected as above and harvested, washed with PBS twice, then fixed in 70% ethanol for 30 minutes at 4 °C. The cells were treated with 1U DNase-free RNase in 1 ml of PBS for 30 minutes at 37 °C, and finally, resuspended in 0.05 mg ml^{-1} propidium iodide (made as a 10× stock in PBS, and cells were analysed by flow cytometry using a FACScan model (Becton Dickinson). Fluorescence measurements were accumulated to form a distribution curve of DNA content. Fluorescence events due to debris were subtracted before analysis.

Western blot detection of p21. Cells (3–5 × 10^6) were harvested at the time points indicated, lysed with 1 ml of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2% SDS, 0.1% bromphenol blue, 10% glycerol, and boiled for 5 minutes. The samples were finally spun at 10,000 r.p.m. for 5 minutes, and supernatants were collected. Samples (20 µl) were loaded into 15% SDS-PAGE and blotted onto nitrocellulose membranes. p21 protein was visualized using an anti-peptide rabbit polyclonal antibody (Santa Cruz) together with an anti-rabbit horseradish peroxidase secondary antibody and subsequent enhanced chemiluminescent detection (ECL) (Amersham).

Gene transfer of *p21*. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. The recombinant adenoviral vector, ADV-p21, was constructed by homologous recombination between sub360 genomic DNA, an Ad5 derivative with a deletion in the E3 region, and a p21 expression plasmid, pAd-p21. These recombinant adenoviral vectors have sequences in the E1A and E1B region deleted, impairing the ability of this virus to replicate and transform nonpermissive cells. Briefly, the pAd-p21

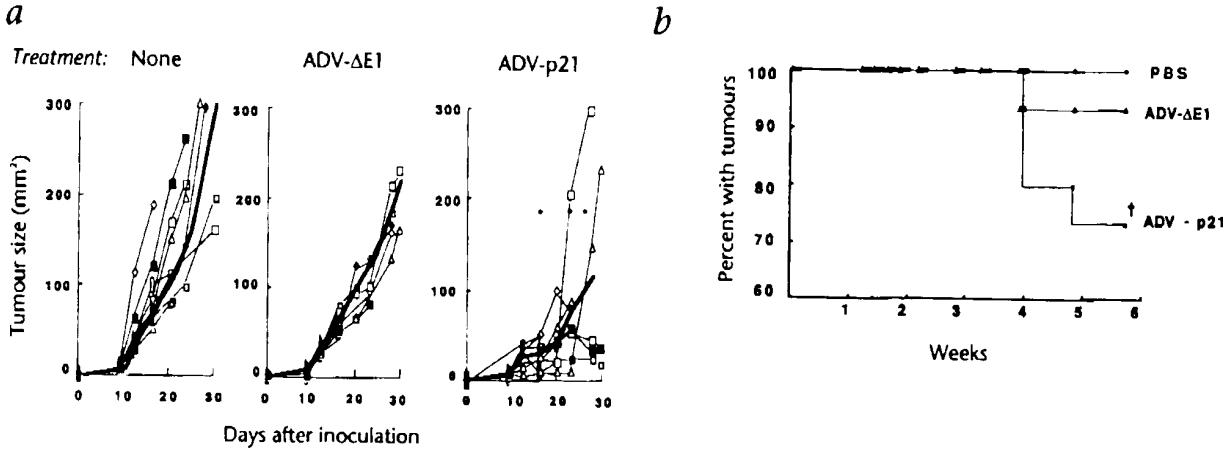

Fig. 6 Introduction of ADV-p21 into established Renca tumour cells *in vivo* inhibits tumour growth. *a*, BALB/c mice bearing Renca tumours were injected with PBS ($n = 7$), ADV-ΔE1 ($n = 7$), or ADV-p21 ($n = 7$) on days 9–13 after tumour inoculation. The bold line indicates the average tumour size for all animals in each group. The asterisks indicate statistically significant differences for ADV-p21-treated animals compared with controls treated with PBS or ADV-ΔE1 on the indicated days (day 16: ADV-p21 vs. PBS, $P \leq 0.003$; ADV-p21 vs. ADV-ΔE1, $P \leq 0.003$; day 20: ADV-p21 vs. PBS, $P \leq 0.004$; ADV-p21 vs. ADV-ΔE1, $P \leq 0.044$; day 23: ADV-p21 vs. PBS, $P \leq 0.041$). *b*, Percentage of animals with pre-existing tumours determined to be tumour-free after treatment is shown as a function of time. The symbol (†) indicates a statistically significant difference for ADV-p21 vs. PBS and ADV-ΔE1 ($P = 0.03$ by Wilcoxon rank sum test). All mice with no evident tumours showed long-term survival (≥40 days). Adenoviral vectors were concentrated by cesium chloride density centrifugation (10^{10} PFU ml^{-1}) and injected intratumourally (0.05 cc). Tumour diameter was measured in two perpendicular dimensions using callipers.

plasmid was prepared by introducing the *Nru*I and *Dra*III fragment from pRc/CMV-p21, kindly provided by D. Beach and G. Hannon[9,13] into the *Bgl*II site of pAd-*Bgl*II (ref. 40), which had the left-hand sequence of Ad5 genome, but not E1A and E1B. Virus was prepared as described previously[41]. The structure of these viruses was confirmed by Southern blotting (unpublished data). All recombinant viruses were propagated in 293 cells and purified as described[40]. Cesium chloride-purified virus was dialysed against PBS, and diluted for storage in 13% glycerol-PBS solution to yield a final concentration of 1–3 $\times 10^{12}$ viral particles per millilitre (0.8–5 $\times 10^{10}$ PFU ml^{-1}). All stocks were sterilized with a 0.45 μm filter and evaluated for the presence of replication-competent adenovirus by infection at a multiplicity of infection (MOI) of 10 onto 3T3 cells. None of the stocks used in these experiments yielded replication-competent virus.

The eukaryotic expression plasmid, pRc/RSV p21, was prepared by introduction of the p21 cDNA from pRc/CMV-p21 into pRc/RSV (Invitrogen), and transfection of 293 cells performed by using calcium phosphate transfection (N.D.P. *et al.*, manuscript submitted).

Bystander assay. U373 human glioblastoma cells, kindly provided by K. Murazko and P. Kish, were infected with ADV-p21 (MOI 200). One day later, cells were trypsinized, counted, and mixed with the indicated number of uninfected U373 cells. 10,000 cells for each mixed population were plated into a 96-well disk. Five days later, the MTT assay[42] was performed to determine the proliferation rate of these cell populations.

Acknowledgements

We gratefully acknowledge David Beach and Gregory Hannon for providing p21 vectors and for helpful discussion, Mark Cameron and A. Chang for providing UM-316 cells, J.A. Stein for help in figure preparation and statistical analysis, K. Murazko and P. Kish for U373 cells, D. Gschwend for manuscript preparation, and K.A. Carter for graphic arts. This work was supported in part by grants from the Public Health Service (P01 CA59327 G.J.N., HL43757, DK42760, E.G.N.) and the American Heart Association. E.G.N. is an Established Investigator of the American Heart Association. N.D.P. is the recipient of a Scholar Award from the American Foundation for AIDS Research (AmFAR) made in memory of James Hoese.

RECEIVED 25 APRIL; ACCEPTED 25 AUGUST 1995

1. King, R.W., Jackson, P.K. & Kirschner, M.W. Mitosis in transition. *Cell* 79, 563–571 (1994).
2. Heichman, K.A. & Roberts, J.M. Rules to replicate by. *Cell* 79, 557–562 (1994).
3. Sherr, C.J. G1 phase progression: Cycling on cue. *Cell* 79, 551–555 (1994).
4. Nurse, P. Ordering S phase and M phase in the cell cycle. *Cell* 79, 547–550 (1994).
5. Hunter, T. & Pines, J. Cyclins and cancer II: Cyclin D and CDK inhibitors come of age. *Cell* 79, 573–582 (1994).
6. Peter, M. & Herskowitz, I. Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell* 79, 181–184 (1994).
7. Chang, F. & Herskowitz, I. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* 63, 999–1011 (1990).
8. Zhang, H., Hannon, G.J. & Beach, D. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev.* 8, 1750–1758 (1994).
9. Xiong, Y. *et al.* p21 is a universal inhibitor of cyclin kinases. *Nature* 366, 701–704 (1993).
10. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. & Elledge, S.J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75, 805–816 (1993).
11. Polyak, K. *et al.* Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78, 59–66 (1994).
12. Toyoshima, H. & Hunter, T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 78, 67–74 (1994).
13. Serrano, M., Hannon, G.J. & Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366, 704–707 (1993).
14. Hannon, G.J. & Beach, D. $p15^{INK4B}$ is a potential effector of TGF—induced cell cycle arrest. *Nature* 371, 257–261 (1994).
15. el-Deiry, W.S. *et al.* WAF1, a potential mediator of p53 tumor suppression. *Cell* 75, 817–825 (1993).
16. Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M. & Smith, J.R. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Expl. Cell Res.* 211, 90–98 (1994).
17. Gu, Y., Turck, C.W. & Morgan, D.O. Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature* 366, 707–710 (1993).
18. Li, Y., Jenkins, C.W., Nichols, M.A. & Xiong, Y. Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene* 9, 2261–2268 (1994).
19. el-Deiry, W.S. *et al.* WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* 54, 1169–1174 (1994).
20. Di Leonardo, A., Linke, S.P., Clarkin, K. & Wahl, G.M. DNA damage triggers a prolonged p53-dependent G_1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* 8, 2540–2551 (1994).
21. Dulic, V. *et al.* p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* 76, 1013–1023 (1994).
22. Li, R., Waga, S., Hannon, G.J., Beach, D. & Stillman, B. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* 371, 534–537 (1994).
23. Li, C.Y., Suardet, L. & Little, J.B. Potential role of WAF1/Cip1/p21 as a mediator of TGF-beta cytoinhibitory effect. *J. biol. Chem.* 270, 4971–4974 (1995).
24. Symonds, H. *et al.* p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. *Cell* 78, 703–711 (1994).
25. Chen, P.L., Scully, P., Shew, J.Y., Wang, J.Y. & Lee, W.H. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* 58, 1193–1198 (1989).
26. Kato, J.T., Matsushimi, H., Hiebert, S.W., Ewen, M.E. & Sherr, C.J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* 7, 331–342 (1993).
27. Hu, Q.J., Lees, J.A., Buchleovich, K.J. & Harlow, E. The retinoblastoma protein physically associates with the human cdc2 kinase. *Molec. cell. Biol.* 12, 971–980 (1992).
28. Lin, B.T.-Y., Gruenwald, S., Morla, A.O., Lee, W.-H. & Wang, J. Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. *EMBO J.* 10, 857–866 (1991).
29. Ewen, M.E. *et al.* Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* 73, 487–497 (1993).
30. Riley, D.J., Lee, E.Y. & Lee, W.H. The retinoblastoma protein, more than a tumor suppressor. *Annu. Rev. Cell Biol.* 10, 1–29 (1994).
31. Chellappen, S.P., Hiebert, S., Mudryi, M., Horowitz, J.M. & Nevins, J.R. The E2F transcription factor is a cellular target for the Rb protein. *Cell* 65, 1053–1061 (1991).
32. Dynlacht, B.D., Flores, O., Lees, J.A. & Harlow, E. Differential regulation of E2F transactivation by cyclin/CDK2 complexes. *Genes Dev.* 8, 1722–1786 (1994).
33. Schneider, K.R., Smith, R.L. & O'Shea, E.K. Phosphate-regulated inactivation of the kinase PH080-PH085 by the CDK inhibitor PH081. *Science* 266, 122–126 (1994).
34. Kaffman, A., Herskowitz, I., Tjian, R. & O'Shea, E.K. Phosphorylation of the transcription factor PH04 by a cyclin-CDK complex, PH080-PH085. *Science* 263, 1153–1156 (1994).
35. Christman, J.K., Mendelsohn, N., Herzog, D. & Schneiderman, N. Effect of 5-azacytidine on differentation and DNA methylation in human promyelocytic leukemia cells (HL-60). *Cancer Res.* 43, 763–769 (1983).
36. Samid, D., Shack, S. & Sherman, L.T. Phenylacetate: A novel nontoxic inducer of tumor cell differentation. *Cancer Res.* 52, 1988–1992 (1992).
37. Pierce, G.B. & Speers, W.C. Tumor as caricatures of the process of tissue renewal: Prospects for therapy by directing differentation. *Cancer Res.* 48, 1996–2006 (1988).
38. Leftwich, J.A. & Hall, R.E. Enhancement of phorbol ester-induced HL-60-mediated cytotoxicity by retinoic acid, dimethyl sulfoxide and 5-azacytidine. *Cancer Res.* 46, 3789–3792 (1986).
39. Chen, S.H. *et al.* Combination gene therapy for liver metastasis of colon carcinoma *in vivo*. *Proc. natn. Acad. Sci. U.S.A.* 92, 2577–2581 (1995).
40. Davidson, B.L., Allen, E.D., Kozarsky, K.F., Wilson, J.M. & Roessler, B.J. A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. *Nature Genet.* 3, 219–223 (1993).
41. Ohno, T. *et al.* Gene therapy for vascular smooth muscle cell proliferation following arterial injury. *Science* 265, 781–784 (1994).
42. Mosman, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. immunol. Methods* 65, 55–63 (1983).
43. Ito, S., Wakamatsu, K. & Ozeki, H. Spectrophotometric assay of eumelanins in tissue samples. *Anal. Biochem.* 215, 273–277 (1993).
44. Gorczyca, W. *et al.* Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. *Leukemia* 7, 659–670 (1993).